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TITLE: Identification and Characterization of Distinct Apoptotic Pathways in Cancer Cells Activated in Response to Treatment with Different Anti-Cancer Agents

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## INTRODUCTION

Apoptosis is a programmed form of cell death that plays an important role in malignancy by shifting the balance from tumor proliferation to its regression. Anticancer drugs act by activating apoptosis in tumor cells. Mutations in apoptotic pathways can lead to anticancer drug resistance and therefore can promote tumor progression. Our lab is working to elucidate the molecular mechanisms of apoptosis in oncogenically-transformed primary Mouse Embryo Fibroblasts (MEFs). We have chosen this model system because of the fact that it lacks mutations and alterations that are common to immortal cell lines; of the ability to use genetic approach to study apoptosis (availability of knock-out mouse lines); of the ease of gene manipulations (retroviral mediated gene transfer technique) in MEFs and the fact that E1A oncogene was shown to induce changes in MEFs similar to those occur in spontaneous tumors. We expect that further insight into mechanisms of programmed cell death in oncogenically-transformed MEFs will provide a fuller understanding of the role of apoptosis in real tumor progression such as breast cancer and will lead to the developing new strategies for anti-cancer therapy.

I am interested in elucidating the role of caspases in apoptosis in MEFs. It has been recently discovered that caspases are essential components of apoptosis machinery. These enzymes may play an important role in drug sensitivity and specific tumor suppression. The goal of my thesis project is to identify caspases that are involved in programmed cell death in primary MEFs, to identify caspases activated during distinct apoptotic programs, to determine the effect of oncogene (E1A) expression on caspases in the cells and finally to establish the role of one particular caspase in distinct forms of apoptosis in oncogenically-transformed MEFs.

## BODY

**Aim 1.** Identification and characterization of distinct apoptotic pathways in E1A/ras-transformed MEFs.

Adriamycin induces apoptosis in p53-dependent manner.

Our laboratory has studied the function of endogenous p53 using null mutant cells derived from p53-knockout mice. Normal MEFs are resistant to cytotoxic drugs, whereas MEFs transformed by E1A and ras (ER/MEFs) are susceptible to apoptosis induced by many anticancer agents. Agents such as  $\gamma$ -radiation or the chemotherapeutic agent Adriamycin induce apoptosis in p53-dependent manner, since p53<sup>+/+</sup> ER/MEFs die but p53<sup>-/-</sup>ER/MEFs are resistant (Fig. 1)

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### TNF- $\alpha$ induces p53-independent apoptosis.

While working at MIT Scott Lowe (unpublished results) has shown that cytotoxic agent tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induces apoptosis in ER/MEFs by p53-independent mechanism (fig. 1). Thus, unlike  $\gamma$ -irradiation, that is effective only on the cells with wild type copy of p53, TNF- $\alpha$  activates cell death both in p53<sup>+/+</sup> and p53<sup>-/-</sup> ER/MEFs. These observations argue that oncogenic transformation by E1A/ras sensitizes MEFs to apoptosis through genetically distinguishable pathways.

### CrmA suppresses apoptosis triggered by TNF- $\alpha$ but not by Adriamycin.

To determine the role of caspases in these distinct forms of apoptosis, I used retroviral-mediated gene transfer to introduce caspase inhibitor CrmA into ER/MEFs. ER/MEFs that were utilized in this experiment were clones of p53<sup>+/+</sup> and p53<sup>-/-</sup> MEFs that were selected after they were co-transfected with E1A and ras expressing vectors. Two clones for each type of cells were infected with CrmA virus and after selection were treated with either Adriamycin, which like  $\gamma$ -irradiation induces p53-dependent apoptosis in ER/MEFs, or TNF- $\alpha$ , to induce p53-independent apoptosis (fig. 2). CrmA protected cells from programmed cell death only when apoptosis was induced by TNF- $\alpha$ , but not by Adriamycin. Since CrmA is a competitive inhibitor for some caspases, this experiment provides preliminary genetic evidence that different caspases function in different apoptotic pathways.

This conclusion was also supported by studies that use a caspase affinity labeling assay developed by Lavina Faleiro and Dr. Yuri Lazebnik at Cold Spring Harbor Laboratory. Briefly, a biotin-linked peptide inhibitor (YVAD or DEVD) that covalently binds only to activated caspases is incubated with lysates from untreated or apoptotic cells. Caspases are visualized by an avidin-based chemiluminescent system after one dimensional SDS-PAGE and transfer to the membrane. The procedure allows visualization of the p20 subunits of active caspases (fig. 3). Altogether the above results suggest the existence of parallel apoptotic pathways in ER/MEFs, that distinct caspases act in these pathways, and that a caspase(s) essential for TNF- $\alpha$  pathway is dispensable for p53-dependent death.

I made several attempts to construct retroviral vector carrying p35 (viral caspase inhibitor) and introduce it into ER/MEFs. All the attempts were unsuccessful, possibly due to toxicity of p35 to the cells. There are several reports from other labs which encounter similar problem.

### Adriamycin and TNF- $\alpha$ induced pathways converge at the point of common caspases activation.

Since molecular weights of the p20 subunits of the caspases are very similar I performed the two-dimensional gel electrophoresis (2D) together with caspase affinity labeling assay to determine whether similar or different sets of caspases are activated during distinct apoptotic programs. 2D blots revealed that treatment of ER/MEFs with adriamycin or TNF- $\alpha$  led to appearance of 7 common activated caspase-specific spots and at least 4 unique for TNF- $\alpha$  pathway spots (fig. 4). These spots can represent different caspases or post-translational modifications of one or several caspases. This data cooperates well with the above conclusions that TNF- $\alpha$  pathway may utilize the unique set of caspases, presumably at the upstream levels of the pathway. One or several of those caspases can be a target for CrmA inhibitor of apoptosis. These results also show that both pathways utilize common set of the caspases. Together these observations support the hypothesis that two pathway may converge somewhere upstream of the step of common caspases activation (fig. 5).

### Synergistic effect of Adriamycin and TNF- $\alpha$ on apoptosis in ER/MEFs.

I observed synergistic effect when p53<sup>+/+</sup> and p53<sup>-/-</sup> ER/MEFs were treated with both drugs (Adriamycin and TNF- $\alpha$ ) simultaneously (fig. 6). p53<sup>-/-</sup>MEFs that were resistant to Adriamycin and sensitive to TNF- $\alpha$  (30% survived), were almost completely killed (only 3% survived) when both drugs were used together. The same were true for p53<sup>+/+</sup>ER/MEFs that were 40% viable after treatment with 0.05  $\mu$ g/ml of Adriamycin, 60% viable after TNF- $\alpha$  treatment and almost completely killed (6% survived) after combinational treatment. The most intriguing result was that CrmA block both TNF- $\alpha$  and combination (TNF- $\alpha$  and Adriamycin) -induced cell death in p53<sup>-/-</sup>ER/MEFs and did not protect p53<sup>+/+</sup>ER/MEFs from any drug and combination of drugs. These results suggest the existence of possible crosstalk between the pathways.

### **Aim 2.** Identification of caspases activated during distinct apoptotic programs in ER/MEFs.

#### Identification of caspases expressed in wild-type E1A/ras/MEFs.

Nine members of mouse caspase family have been identified. I determined which ones are expressed in MEFs. I have established that Caspases-2,3,6,7,8,12 are expressed in primary MEFs at the RNA level (fig. 7). Caspases-2,3,7,8 and 11 can also be detected at protein level (fig. 8). My results show that introduction of E1A oncogene greatly increases protein level of the unprocessed forms of the caspases that were tested. Moreover, caspase induction is p53 independent. I detected little, if any, induction of caspases at mRNA levels. That



argues that this process might occur at the translational or post-translational levels, for example, E1A could somehow alter protein stability of pro-caspases.

E1A oncoprotein is known to sensitize cells to undergo apoptosis following treatment with anticancer agents through a mechanism involving inactivation of retinoblastoma (Rb) protein and stabilization of p53 tumor suppressor. The downstream events of this mechanism remain to be discovered. Anticancer agents ultimately induce apoptosis by activating caspases. Caspases are expressed as latent pro-enzymes and processed to active enzymes during apoptotic cell death. It is feasible that the high levels of pro-caspases that are available for activation by apoptotic agents could predispose cells to apoptosis. Caspase induction by E1A may be a part of the mechanism by which E1A promote chemosensitivity.

Since E1A promotes apoptosis and causes dramatic induction of pro-caspases I decided to focus my future research on mechanism of caspase induction by E1A (see Aim 4).

#### Generation of monoclonal antibodies against mouse caspases expressed in E1A/ras/MEFs.

For Western blots in the above experiments I used specific anti-caspase antibodies that recognize mouse caspases. To obtain these antibodies I have established collaboration with Yuri Lazebnik's Lab, which developed antibodies against certain human caspases. In addition to that I tested human, rat and mouse antibodies that are commercially available. I was lucky enough to find that anti-human antibodies against Caspase-7, developed by Yuri Lazebnik, and Caspase-2 available from Santa Cruz crossreact with mouse Caspase-7 and Caspase-2 accordingly and appeared to be very specific

I also attempted to develop mouse specific anti-caspase antibodies (for Caspase 2,3,6,7,8,11,12) in collaboration with Dr. Walter Fiers's laboratory (University of Ghent, Belgium) that supplied us with purified recombinant p30 subunits of mouse caspases that lack putative prodomains. Although it took almost 9 months I obtained only one specific for Caspase-8 antibody that recognize at least unprocessed 53 and 55kD forms of the pro-caspase 8 on Western blots. I also developed several specific polyclonal sera to Caspase-8 and Caspase-11. Most of the other clones for monoclonal antibodies were unspecific they cross-reacted with other caspases as tested by dot blot analysis with purified caspases. This probably is due to the fact that caspases have very high similarity in protein sequence in their p20 and p10 regions.

#### Establishing the pattern of caspase activation in wild-type E1A/ras/MEFs.

Using specific for p20 or p10 subunits of a particular caspase antibodies I planed to establish which of those caspases that are expressed in ER/MEFs become processed during apoptosis triggered by two anticancer agents known to induce distinct apoptotic programs. This would allow me not only to establish

which caspases participate in apoptosis in our system but also provide insight of whether similar or different set of caspases act in different apoptotic pathways.

Most antibodies that I obtained and tested recognize only unprocessed form of caspases in MEFs. Caspases-3, 7 and possibly 2 were the only ones for which I was able to detect processing during apoptosis in MEFs. I have shown that Caspase-3 is differently processed during Adriamycin and TNF- $\alpha$  induced apoptosis (Fig. 9).

**Aim 3.** Establishing the role of Caspase-2 in distinct forms of apoptosis in oncogenically transformed mouse embryo fibroblasts.

I have established in the above experiments that Caspase-2 is expressed and possibly processed during apoptosis in ER/MEFs. To examine the role of this particular caspase in cell death I proposed to determine the requirement for Caspase-2 for apoptosis in ER/MEFs.

Determination the role of Caspase-2 in different forms of apoptosis in E1A/ras/MEFs.

To understand the importance of Caspase-2 in apoptotic pathways triggered by Adriamycin and by TNF- $\alpha$ , I isolated MEFs from Caspase-2 knockout and wild type embryos. Then I sensitized MEFs to apoptosis by sequential infection with retroviruses expressing the E1A and ras oncogenes.

Next, I exposed the resulting populations to either Adriamycin or mTNF- $\alpha$ . I compared the ability of wild type and knockout populations of ER/MEFs to undergo apoptosis triggered by different stimuli. Then I examined the ability of drug-treated Caspase-2<sup>-/-</sup> cells to display common markers of apoptosis such as DNA laddering and chromatin condensation.

I did not detect any difference in apoptotic response between wild type and knockout cells (Fig. 10). Moreover, Caspase-2 deficient and wild type cells were equally sensitive to  $\gamma$ -radiation, cis-platinum, adenoviral infection, serum starvation and etoposide (fig. 11). I also did not detect any substantial defects of Caspase-2<sup>-/-</sup> cells in their ability to display common markers of apoptosis such as DNA laddering and chromatin condensation (fig. 12, 13). These results together with the observation that Caspase-2 is expressed and possibly processed in MEFs argue for the possible redundancy of function between the caspase members in mouse embryo fibroblasts.

Establishing the pattern of caspase activation in Caspase-2 knockout E1A/ras/MEFs.

To determine the difference in patterns of caspase activation between wild type and Caspase-2<sup>-/-</sup> E1A/ras/MEFs, I used Western blot analysis. My results

demonstrated that processing of Caspase-3 and Caspase-7 in Caspase-2 knockout cells indistinguishable from wild-type cells (data not shown).

In addition to Caspase-2 I also determined the requirement of the Caspase-11 for apoptosis in ER/MEFs. Similarly to caspase-2 I did not find any difference in apoptotic response to Adriamycin and TNF- $\alpha$  of Caspase-11 knockout cells (data not shown).

**Aim 4.** Determination of the mechanism by which the E1A oncogene induces the pro-caspase levels.

My results from Northern blot analysis of caspase expression clearly show that pro-caspase induction by E1A occurs on post-transcriptional level (fig. 7). Moreover this induction is independent on p53 function since E1A oncogene still induce pro-caspases in p53-/- cells (fig. 8).

Determine the regions of the E1A protein that are responsible for caspase induction.

I used several E1A deletion mutants that are known to bind certain cellular factors (fig. 14). I introduced these mutants to wild type MEFs and by Western blot analysis with anti-caspase antibodies I have established that CR2 region of E1A and possibly N-terminal region as well are responsible for caspases upregulation (Fig. 15).

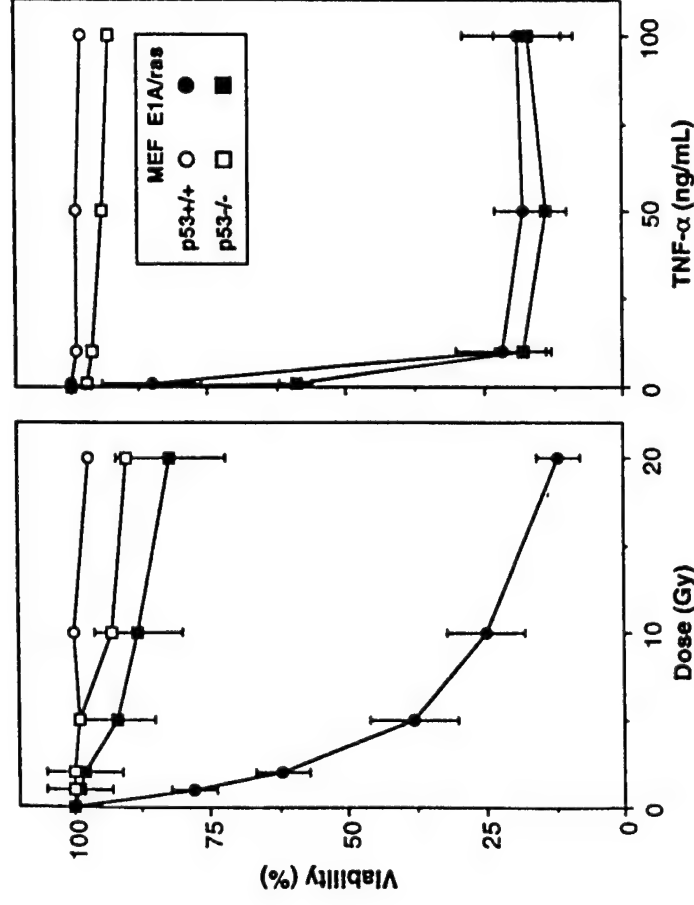
## CONCLUSIONS

1. My data provide evidence supporting the existence of two parallel apoptotic pathways in E1A/ras/MEFs.
2. These two pathways activate common set of the caspases in response to different treatments supporting the hypothesis that these two pathways may converge somewhere upstream of the step of common caspase activation.
3. My results clearly show that TNF- $\alpha$  pathway utilizes unique caspase(s) which possibly act at the upstream levels of the pathway.
4. Treatment of ER/MEFs with adriamycin and TNF- $\alpha$  in combination show that two drugs that are known to trigger apoptosis through distinct mechanisms can synergise in their ability to induce apoptosis.
5. I have established that caspases-2,3,7,8 and 11 are expressed in ER/MEFs both at RNA and protein levels.
6. Caspase-3 and 7 are processed during apoptosis triggered by Adriamycin and TNF- $\alpha$ . My results show that Caspase-3 is differently processed in TNF- $\alpha$  pathway compared to Adriamycin pathway.

7. I have generated at least one specific anti-caspase-8 antibody that recognizes Caspase-8 on Western blots.
8. Caspase-2 is dispensable for apoptosis in ER/MEFs triggered by several apoptotic agents. This argues for possible redundancy of function between caspase members.
9. Introduction of E1A oncogene greatly increases the levels of pro-caspases 2, 3, 7 and 8 in MEFs through a post-transcriptional mechanism. The CR2 domain of E1A is essential for pro-caspase induction, indicating that possible inactivation of Rb by E1A contributes to this effect. In contrast, the p53 tumor suppressor is dispensable for caspase induction, since E1A induces pro-caspase levels in p53<sup>-/-</sup> MEFs.

**Fig. 1**

TNF- $\alpha$  Induced Apoptosis is p53 Independent, while  $\gamma$ -Radiation Induced Apoptosis is p53 Dependent

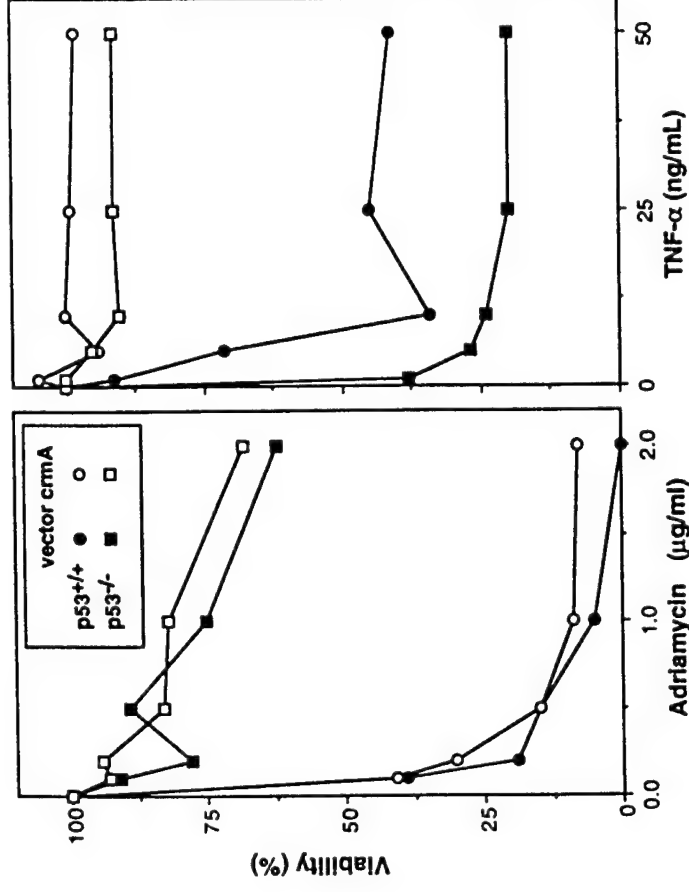


**Conclusion:**

- ER/MEFs are sensitized to apoptosis through genetically distinguishable pathways

**Fig. 2**

CrmA Suppresses Apoptosis Induced by TNF- $\alpha$  but not by Adriamycin

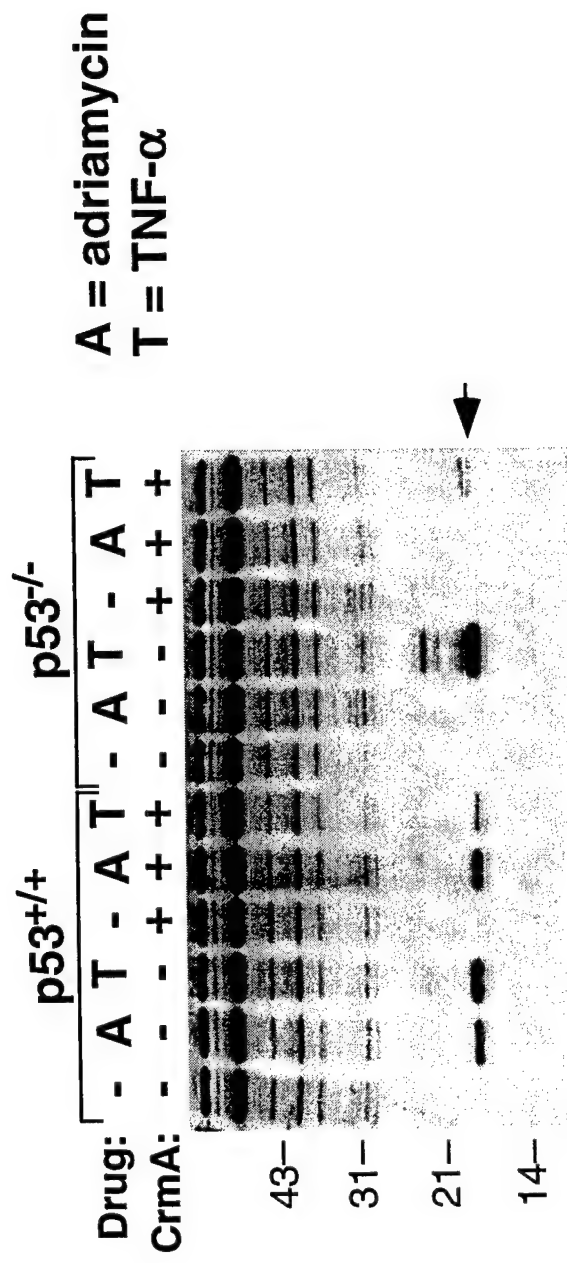


**Conclusions:**

- ILP(s) essential for TNF- $\alpha$  pathway is (are) dispensable for p53 dependent death
- Distinct ILP's function in different apoptotic pathways
- These preliminary results argue for the existence of parallel apoptotic pathways in ER/MEF's

**Fig. 3**

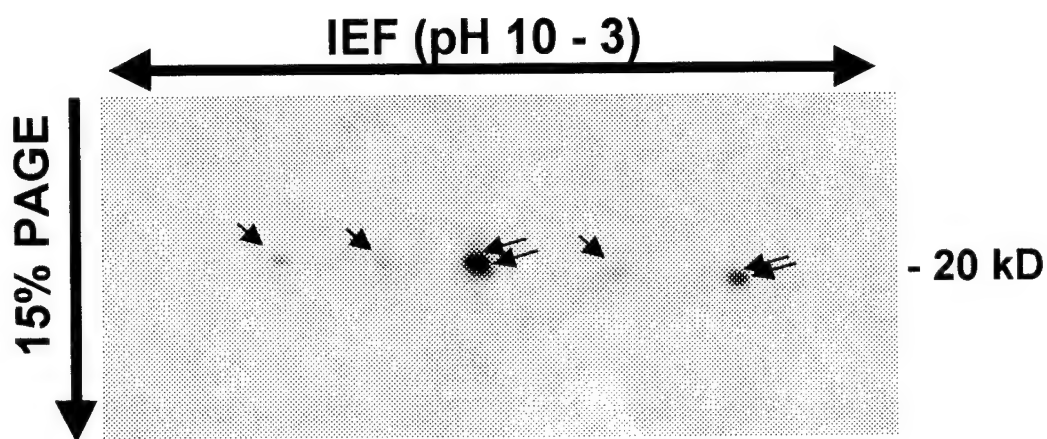
CrmA inhibits ILP activation induced by TNF- $\alpha$  but not  
by adriamycin



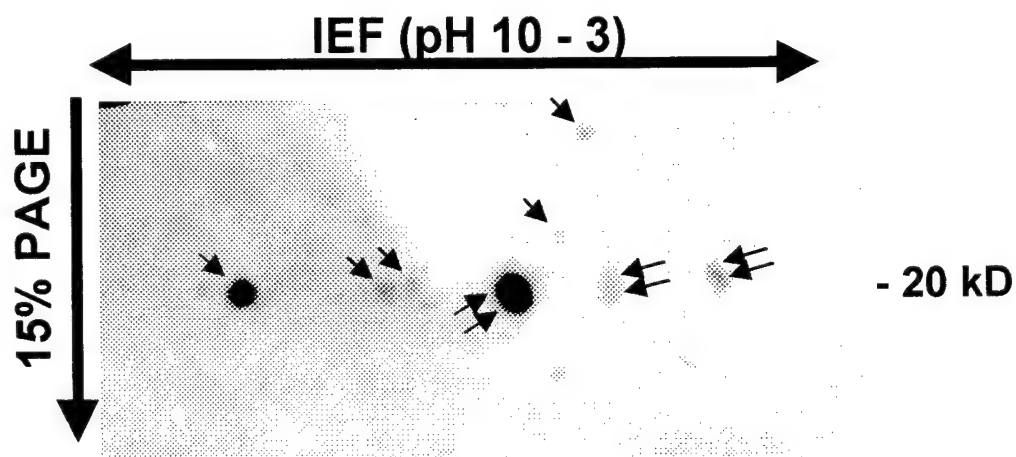
**Fig. 4**

**2D Caspase affinity labeling assay with  
YVAD inhibitor of the treated E1A/ras/MEFs.**

**Adriamycin (0.5  $\mu$ g/ml)**



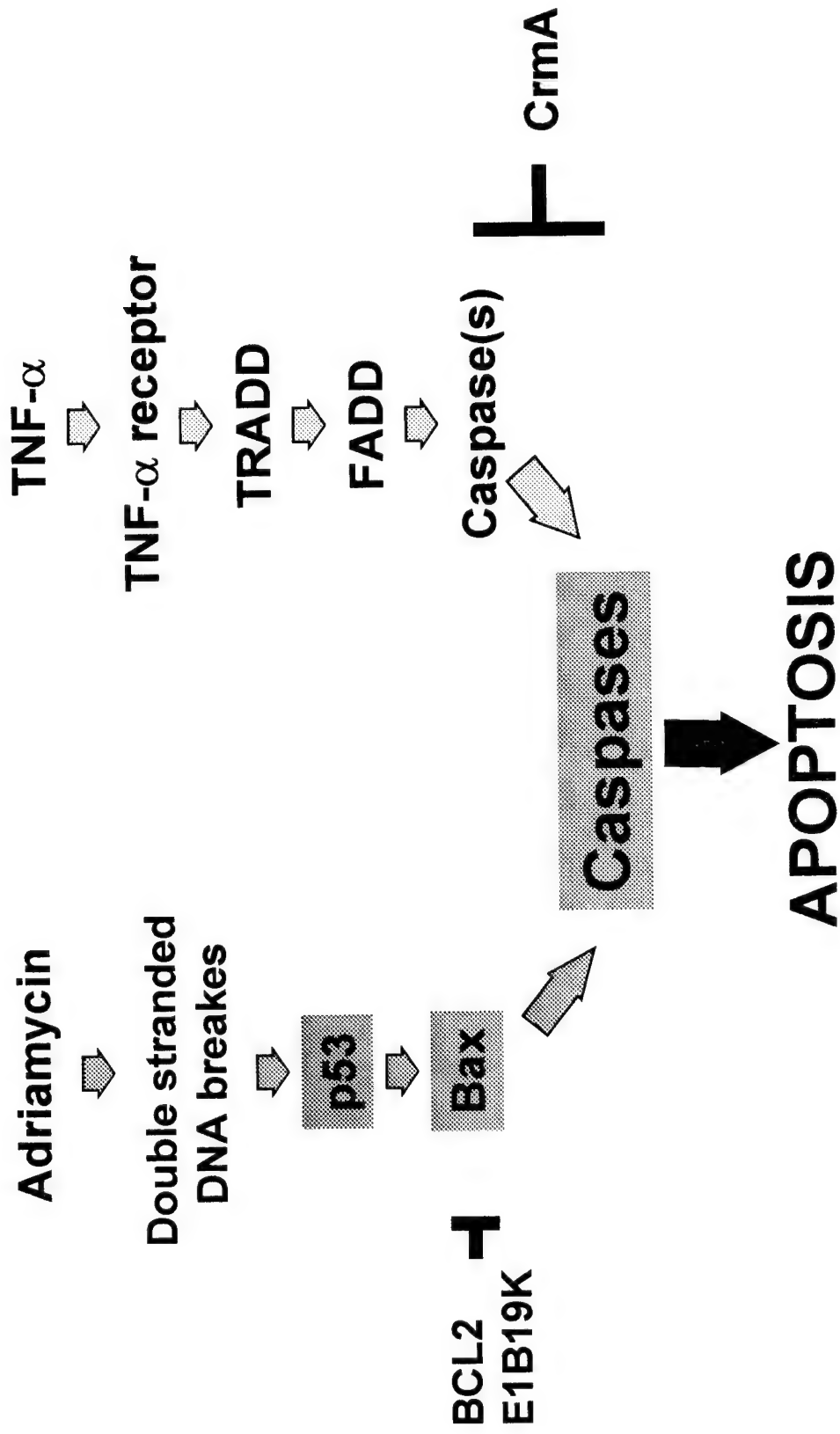
**mTNF- $\alpha$  (25ng/ml)**





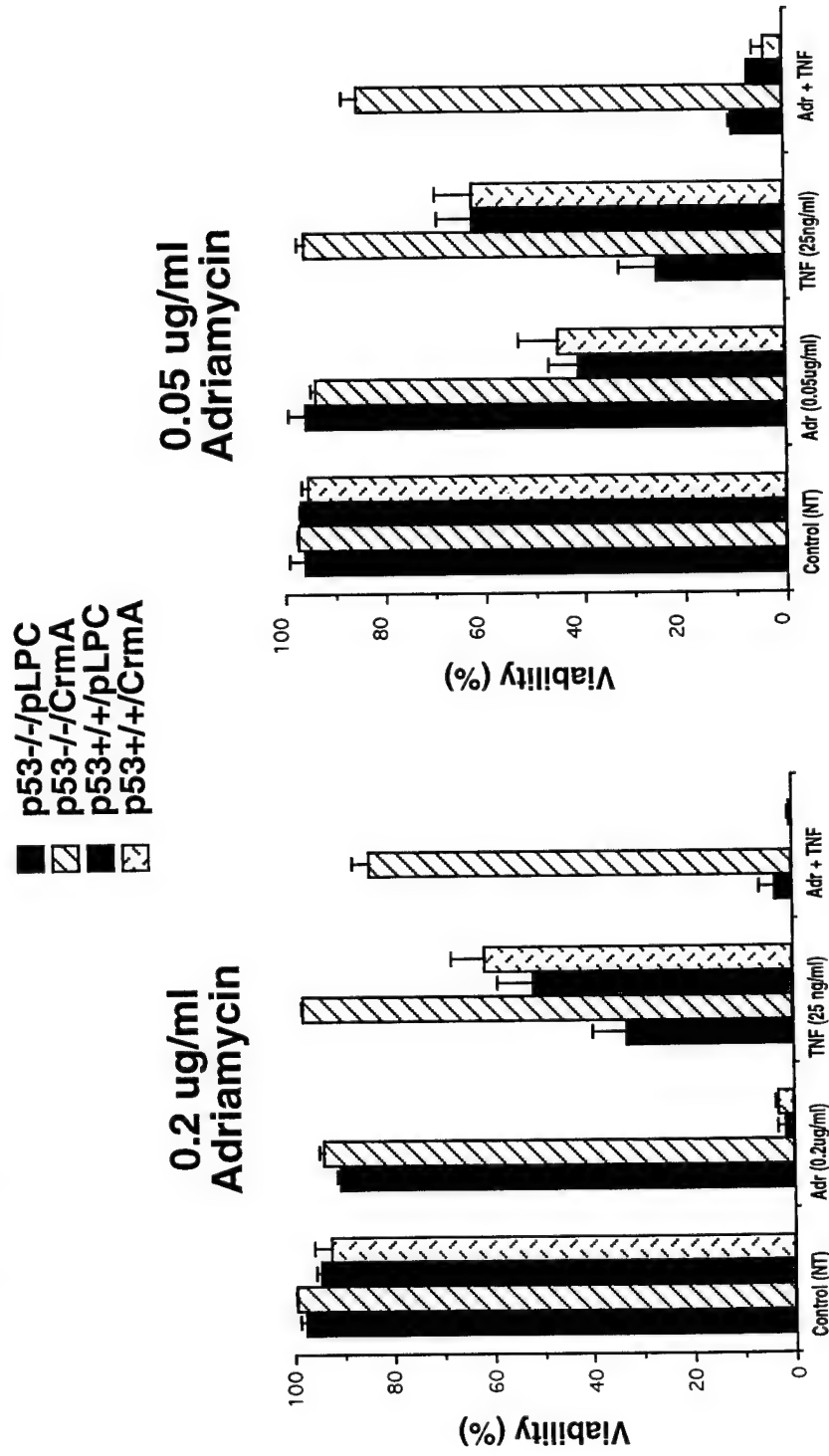
**Fig. 5**

**Model of Parallel Apoptotic Pathways in E1A/Ras/MEFs**

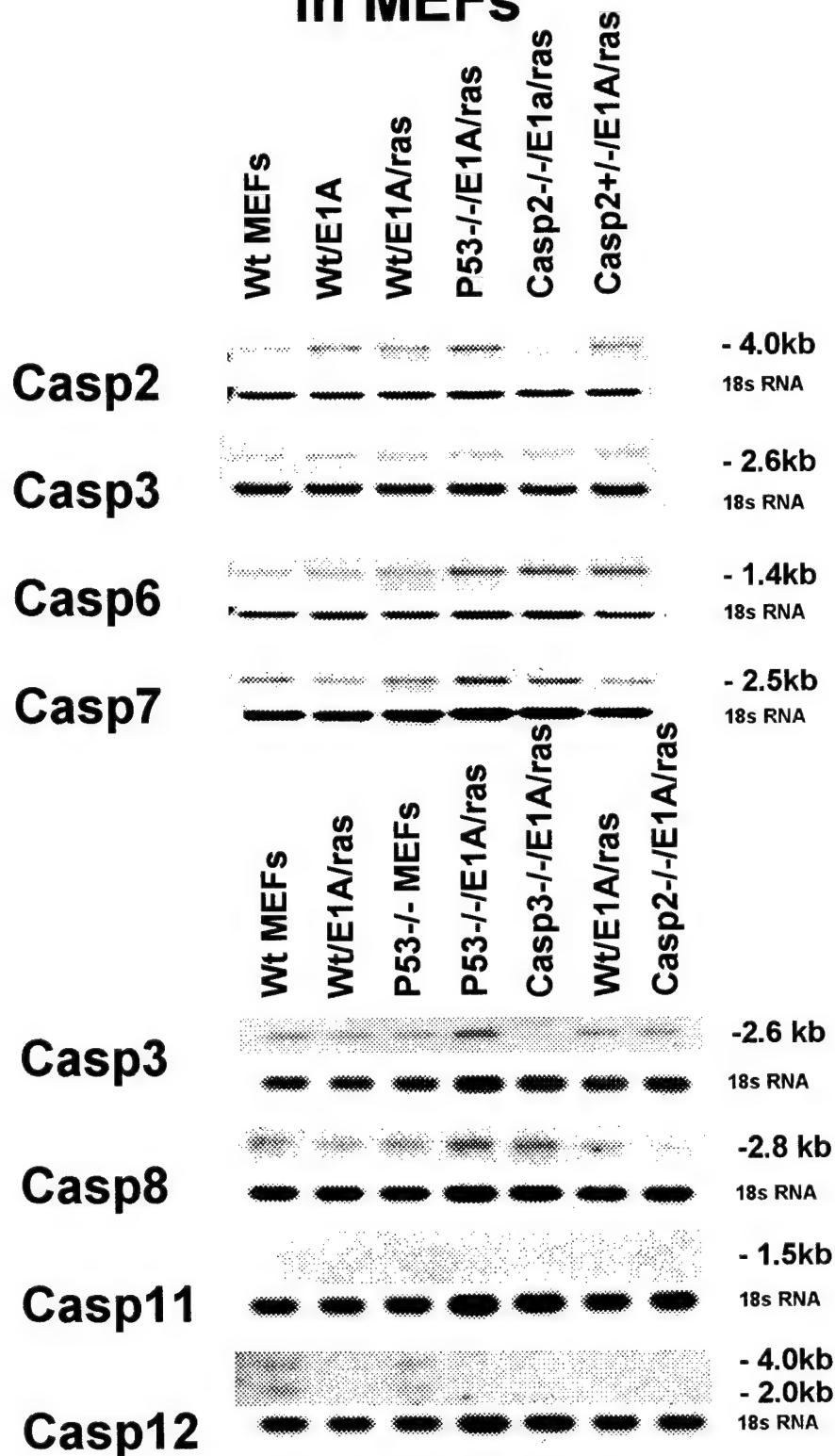


**Fig. 6**

# **Synergistic effect of Adriamycin and TNF- $\alpha$ on apoptosis in ER/MEFs**

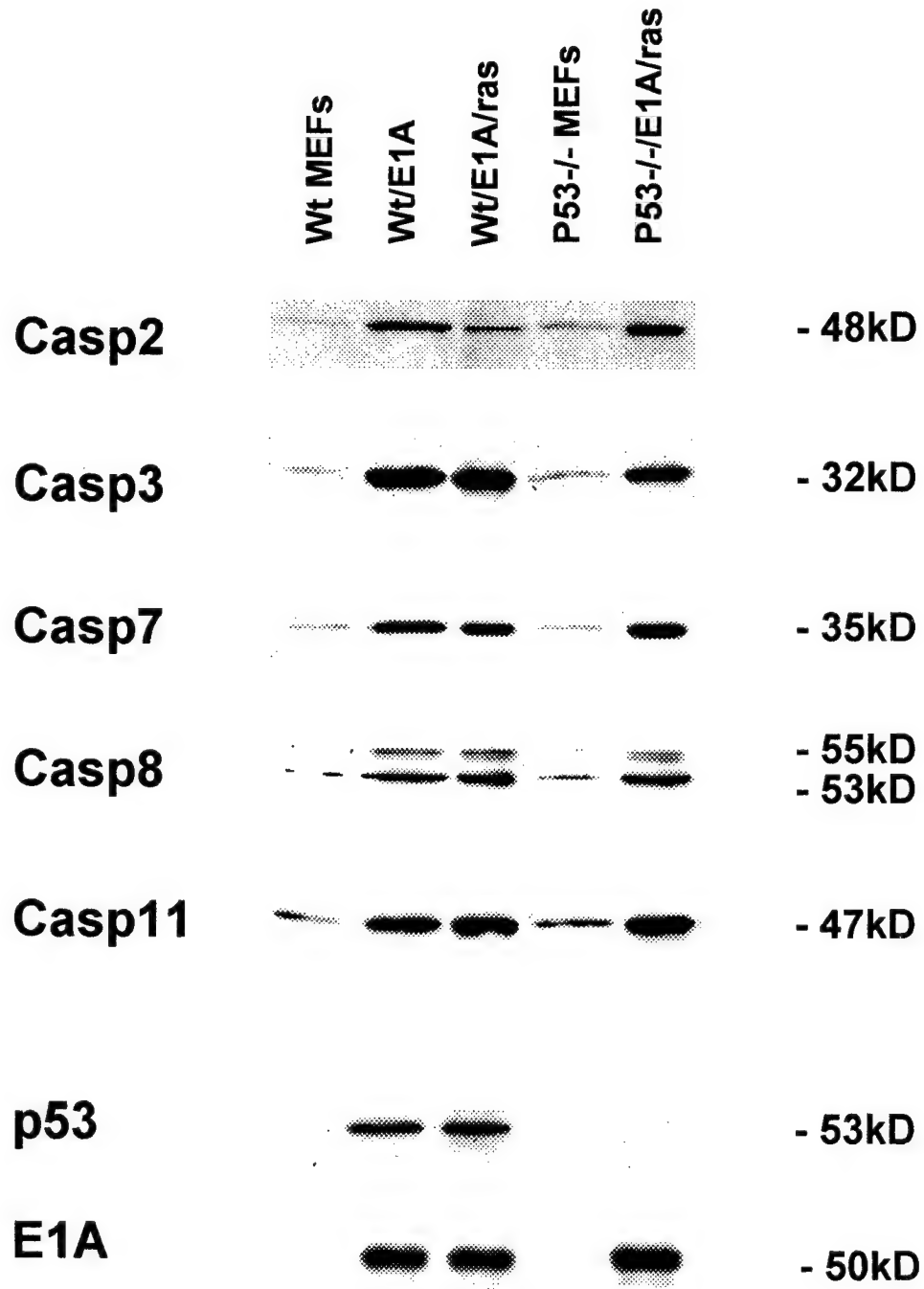


## Northern blot analysis of caspase expression in MEFs



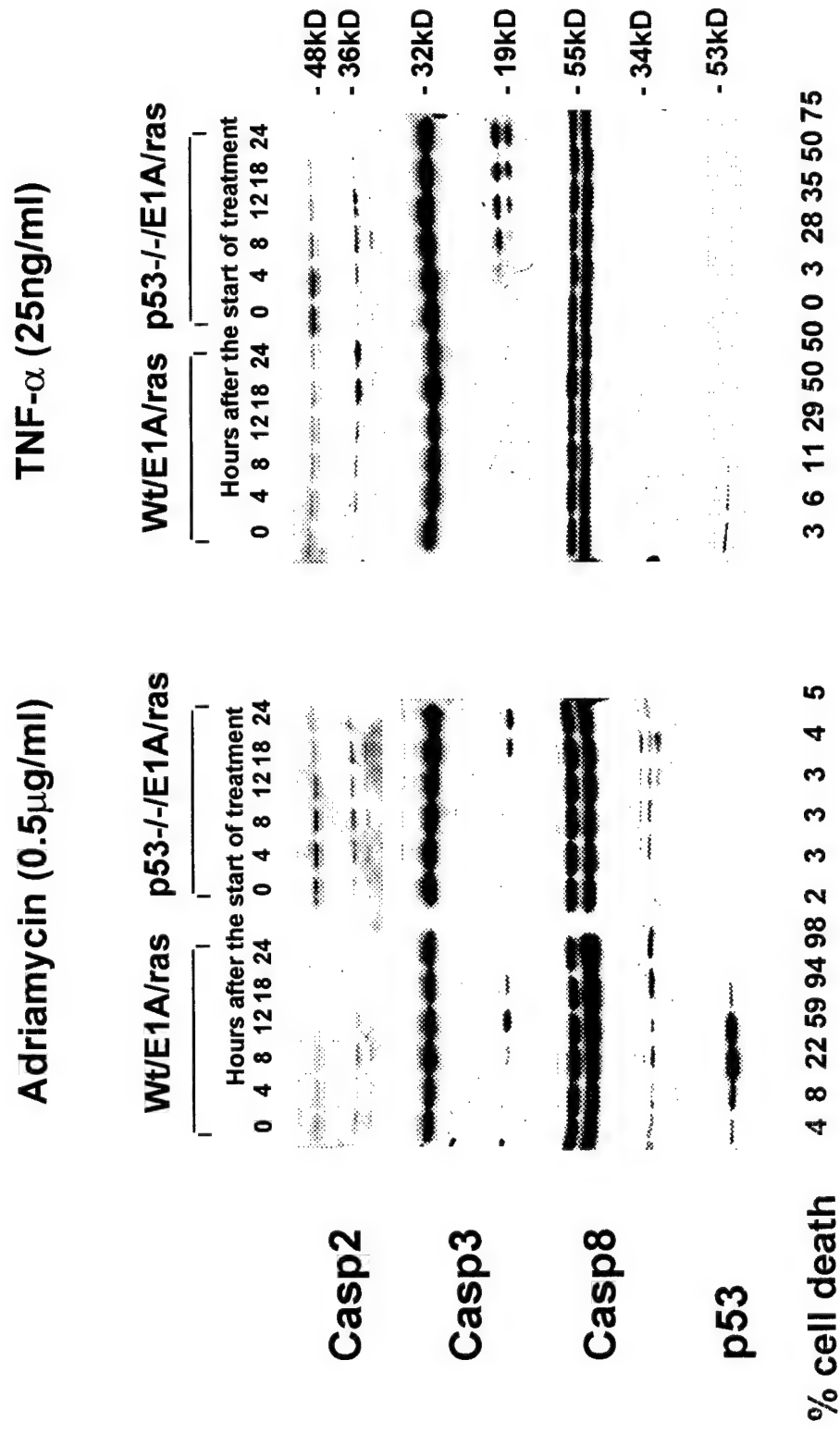
**Fig. 8**

**Western blot analysis of caspase expression  
in untreated MEFs**



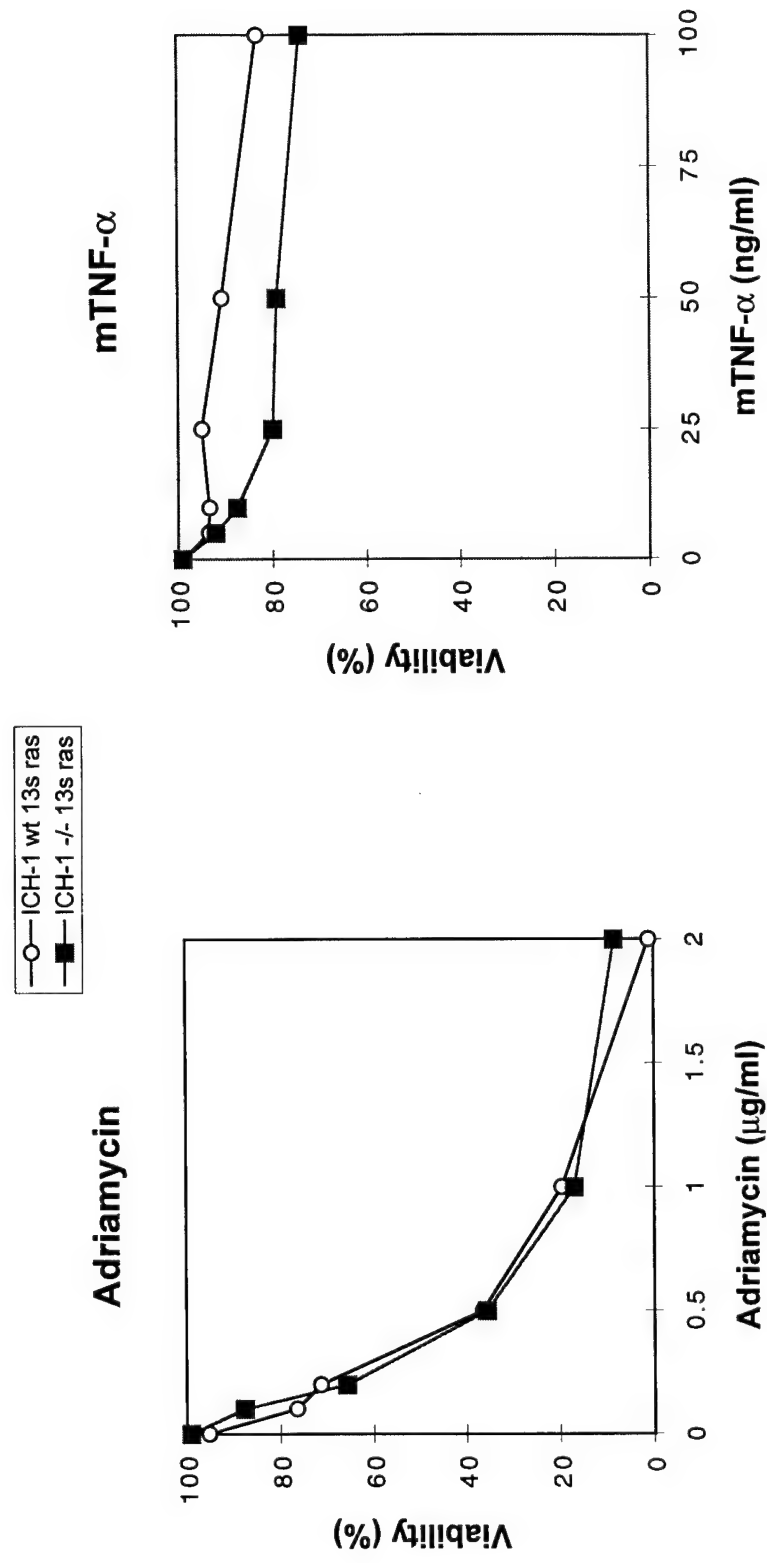
**Fig. 9**

**Western blot analysis of caspase processing in treated  
E1A/ras transformed MEFs**



**Fig. 10**

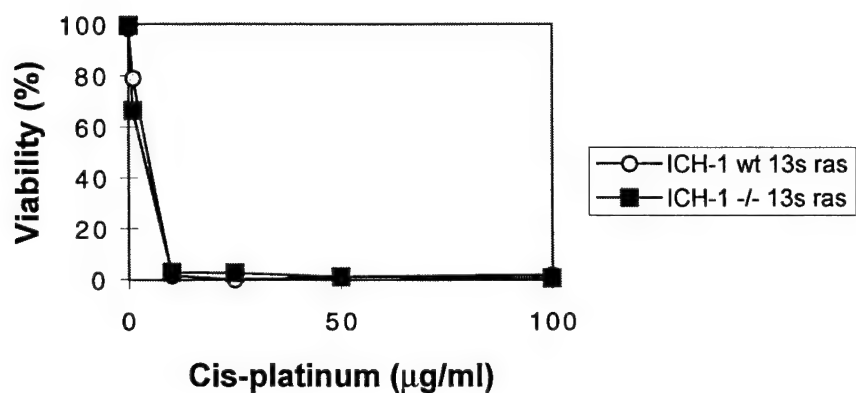
# **Sensitivity of Caspase 2 knock-out cells to Adriamycin or TNF- $\alpha$**



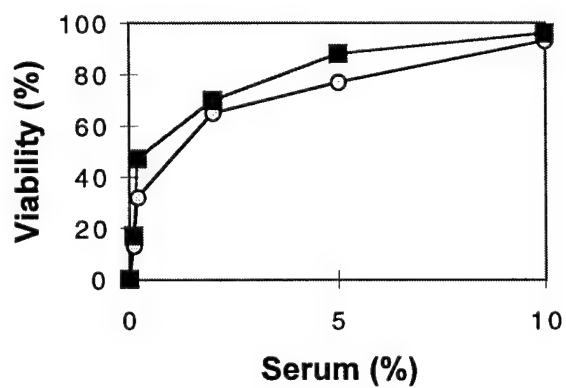
# Sensitivity of Caspase 2 knock-out cells to different treatments

Fig. 11

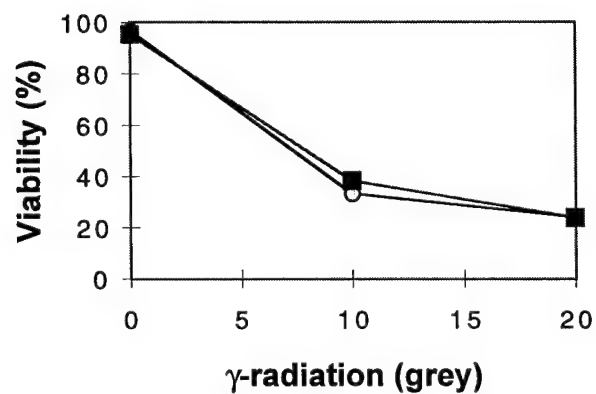
## CIS-PLATINUM



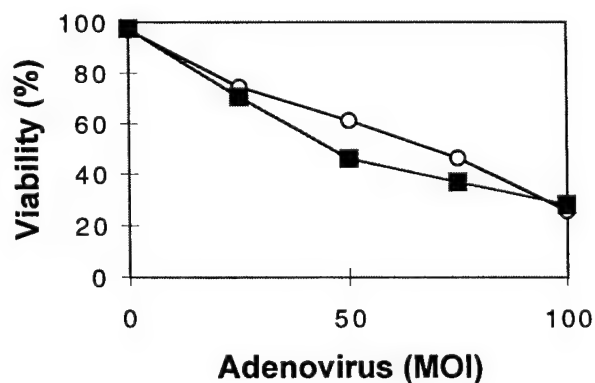
## LOW-SERUM



## γ-RADIATION



## ADENOVIRUS INFECTION



## ETOPOSIDE

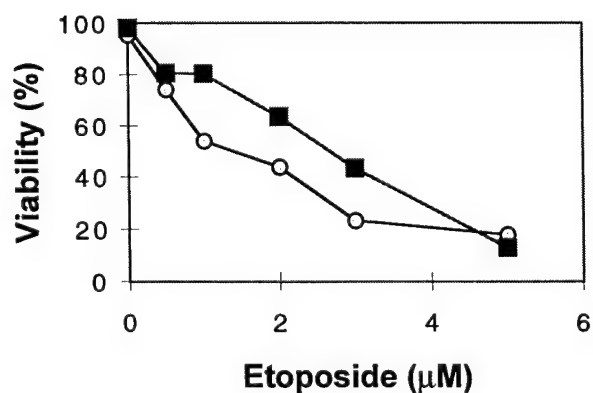


Fig. 12

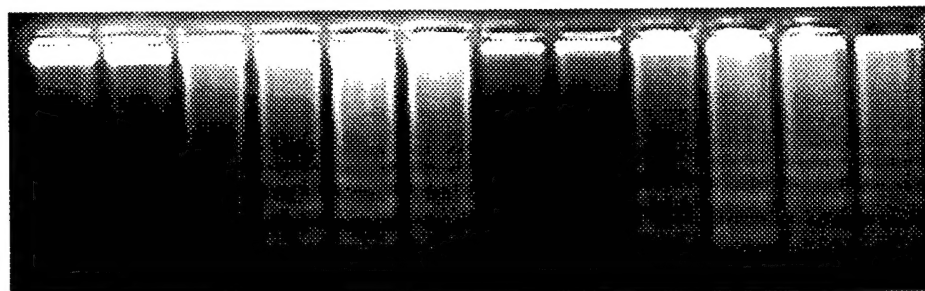
## DNA-laddering analysis of Caspase 2 knock-out E1A/ras/MEFs

### Adriamycin

Wt /E1A/ras

Casp-2-/- /E1A/ras

Hours after the start of the treatment  
0 4 8 12 18 24 0 4 8 12 18 24

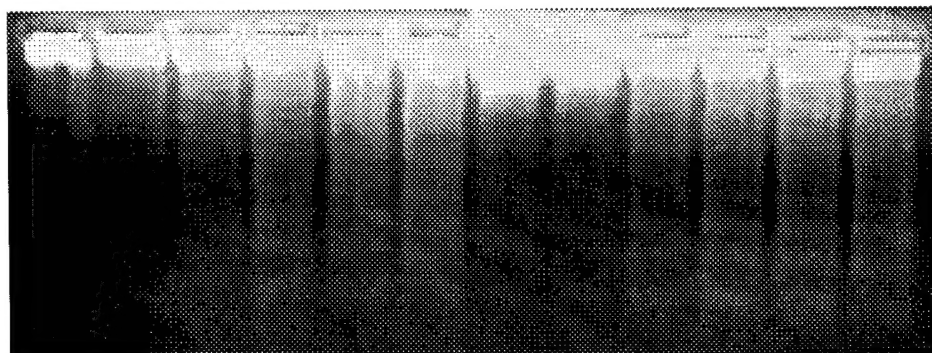


### TNF- $\alpha$

Wt /E1A/ras

Casp-2-/- /E1A/ras

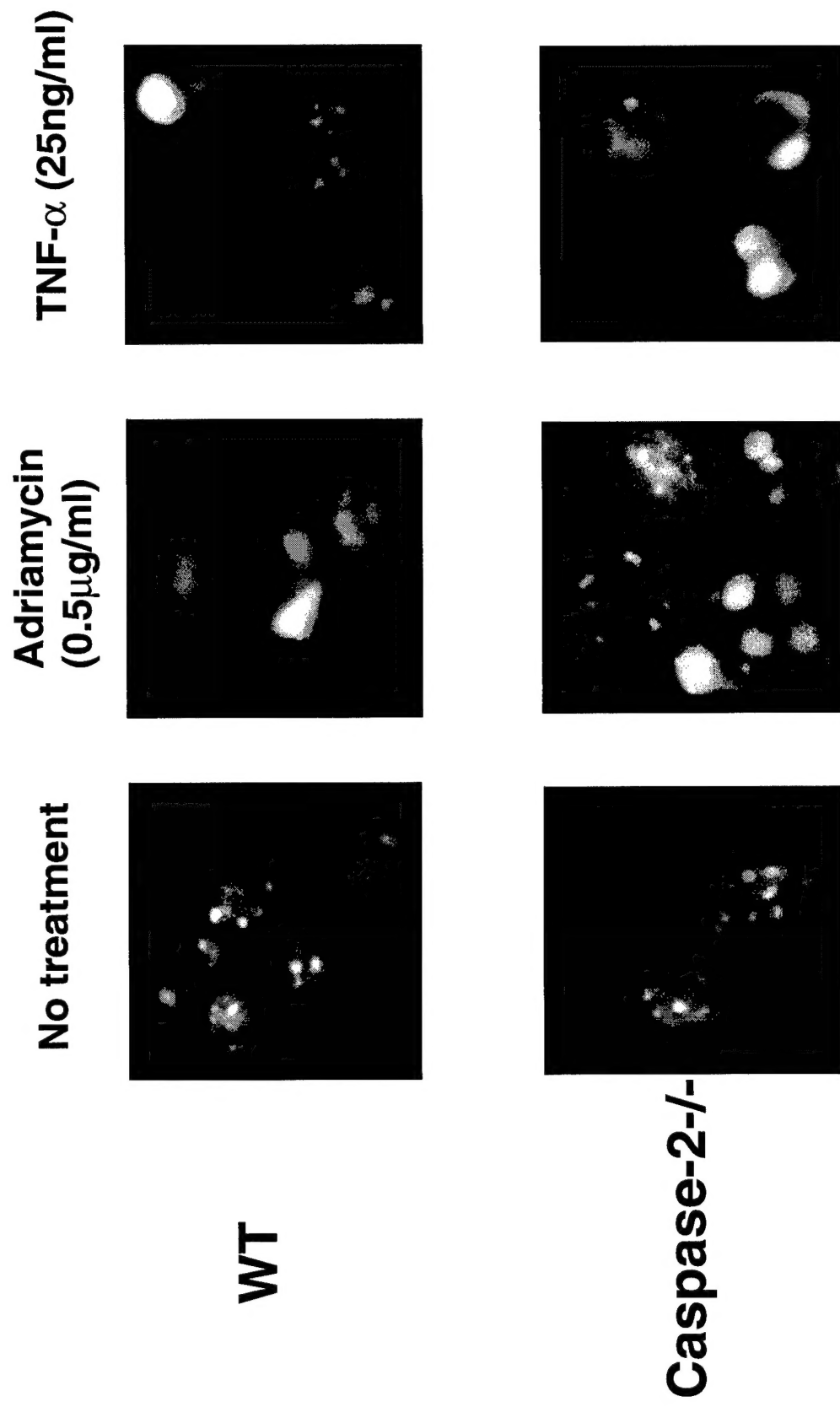
Hours after the start of the treatment  
0 4 8 12 18 24 0 4 8 12 18 24





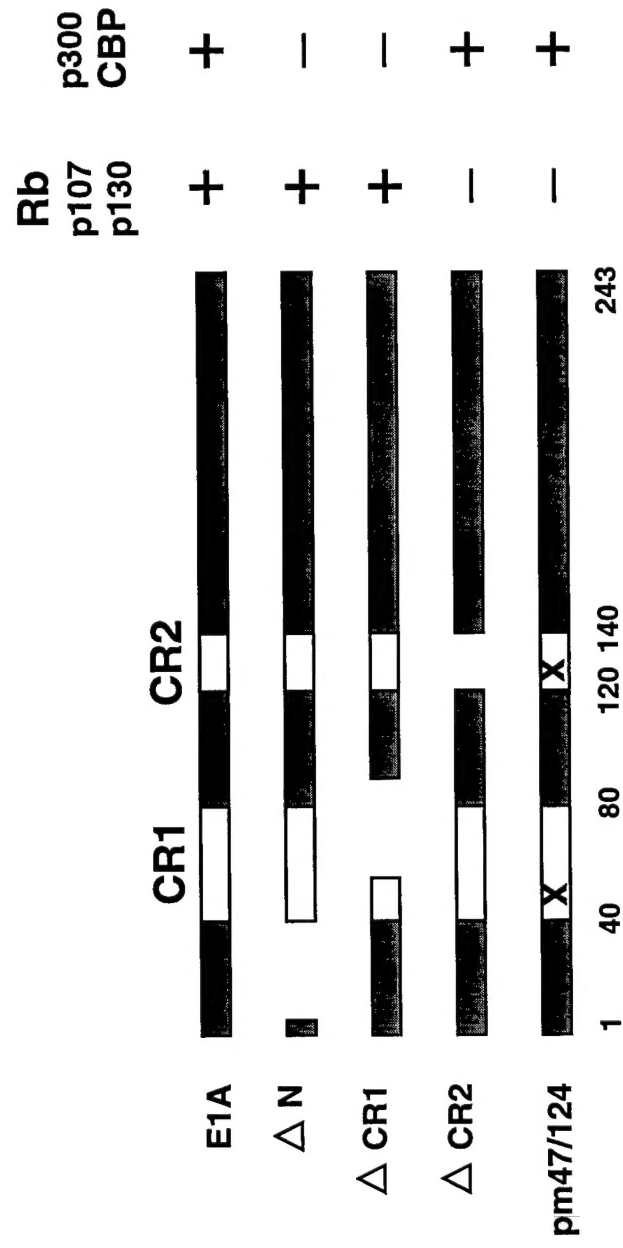
**Fig. 13**

**DAPI analysis of Caspase-2 knock-out E1A/ras/MEFs  
treated with apoptotic agents**



**Fig. 14**

## Structure of E1A mutants



## CR2 region of E1A is required for caspase induction

